Tissue inhibitor of metalloproteinase-1 is increased in the saphenofemoral junction of patients with varices in the leg

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Purpose: **The goal of the present study was to examine the role of matrix metalloproteinase (MMP) activity in the development of varicose changes in the superficial veins of the lower extremity.**

Methods: **Normal-caliber vein segments from the saphenofemoral junction were harvested from patients undergoing saphenous vein ligation for varices and from patients undergoing infrainguinal bypass graft procedures. The activity and quantity of MMPs and their inhibitors (tissue inhibitors of metalloproteinases [TIMPs]) in the vein segments were compared. Vein segments were obtained from 13 patients. Seven patients had varicose disease in the leg, including 6 women and 1 man (average age, 48 years). Six patients had no evidence of varicose disease, including 2 women and 4 men (average age, 59 years). Proteolytic activity was determined with substrate gel zymography, and enzyme content was determined with Western immunoblotting using monoclonal antibodies directed against MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, and α_2-macroglobulin. Signals were quantified by scanning densitometry and normalized to a positive control (densitometric index [DI]). Immunohistochemistry was performed for enzyme localization.**

Results: **Zymography did not detect a difference between groups at loci consistent with the major MMPs; however, a small but significant decrease in proteolytic activity was noted in veins from patients with varices. TIMP-1 is increased in vein segments from patients with varices (DI 0.8 ± 0.1 vs 0.2 ± 0.05, $P < .05$) while MMP-2 levels were decreased (DI 1.5 ± 0.3 vs 0.5 ± 0.1, $P < .05$). Immunohistochemistry localized MMPs to the adventitia of the vein wall.**

Conclusion: **A decrease in proteolytic activity may be responsible for the histological and structural alterations leading to varicose degeneration of superficial lower extremity veins. (J Vasc Surg 1998;28:669-75.)**

Several theories have been advanced to account for the development of varices in the superficial veins of the lower extremity. The most widely held belief is that valvular incompetence in the greater saphenous vein results in venous hypertension and eventual dilatation,[1] and the near-constant association of saphenous incompetence with varicose disease appears to support this hypothesis. Other theories implicating hemodynamic stress in the development of varicose veins include the presence of abnormal arteriovenous communications resulting in venous hypertension[2] and excessive turbulence due to dilated vasa vasorum.[3] Finally, primary disease of the vein wall, in particular with regard to connective tissue metabolism, has been postulated to lead to varicose degeneration.[4] Although circumstantial evidence supporting these theories has been reported, there is a paucity of direct evidence to support them.

The collagen content in varicose veins is increased.[5] Histologic studies have revealed an accumulation of connective tissue within the muscle layers of varicose veins, with disruption of the muscular bundles.[6,7] Smooth muscle cells in varicose veins have

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been noted to contain vacuoles of degraded collagen fibrils, suggesting that these cells contribute to the metabolism of collagen and to the structural alterations noted in varicose disease.[4] Conversely, the elastin content of varicose veins is decreased compared with normal veins. Morphometric studies both in vitro and in vivo have demonstrated a decrease in the elastic properties of varicose veins and suggest that abnormalities of the vein wall precede valvular incompetence in the pathogenesis of varicose veins.[8,9]

Enzymatic activity in vein walls has been studied with respect to tissue remodeling. Decreases in the fibrinolytic activity of calf veins have been reported, whereas an increase in tissue plasminogen activator in the region of the groin has been noted.[10] Elastase activity, however, was not different between normal and varicose veins in a study by Gandhi et al,[5] leading these investigators to exclude enzymatic matrix degradation in the etiology of varicose veins. However, the activity of metalloproteinases (proteolytic enzymes essential in the breakdown and remodeling of connective tissue) in varicose veins has not been investigated. The purpose of this study was to evaluate the proteolytic activity and expression of matrix metalloproteinases (MMPs) in veins from normal patients and patients with lower extremity varicose veins to elucidate the role of these enzymes in the development of varices.

METHODS

Patients and vein samples. After this protocol was approved by the Human Research Committee at our institution, small portions of saphenous vein without obvious varicose changes were collected from the saphenofemoral junction of 13 patients. Seven patients had vein stripping for symptomatic varicose disease located in the distal extremity, including 6 women and 1 man (average age, 48 years). Of these patients, 4 had preoperative duplex examination demonstrating incompetence of the greater saphenous vein at the level of the groin in 3. There was no evidence of arterial insufficiency in any of the patients with varices, none of these patients had diabetes, 1 had a history of coronary artery disease, and 5 had a history of tobacco abuse.

Among the 6 patients who had vein harvested for bypass of infrainguinal occlusive disease, there were 2 women and 4 men (average age, 59 years). There was no evidence of varicose disease at operation in any of these patients, and duplex mapping of the saphenous vein in 2 patients did not demonstrate reflux. Bypass was performed for rest pain in 3 patients and for healing of tissue loss in 3. The ankle-brachial index was 0.4 or less in 4 patients and was not able to be obtained because of calcific tibial artery disease in the remaining 2. Risk factors for coronary artery disease were more common in this group and included a history of diabetes in all 6 patients, coronary artery disease in 4 patients, and tobacco abuse in 5 patients.

Protein preparation. Tissue specimens were frozen in liquid nitrogen at the time of the harvest and placed at –80°C. For analysis, 100-mg to 500-mg pieces of vein wall were thawed and gently agitated in phosphate-buffered saline (PBS) to remove residual blood. A 3-mm segment of vein was taken from the specimen and flash-frozen in OCT mounting solution for histological evaluation. All manipulations were done at 4°C to prevent protein degradation. The remaining samples were minced, flash-frozen in liquid nitrogen, and pulverized to a fine powder. The powdered vein wall then was resuspended in a 2 mol/L sodium chloride/0.05 mol/L Tris/0.1% Brij 35 (v/v) solution at pH 7.5 (0.2 mL of buffer, 100-mg sample). The samples were then centrifuged at 10,000 *g* at 4°C for 1 hour, the supernatant was collected, and protein concentrations were determined according to the method of Bradford.[11]

As recommended by the manufacturer of the antibodies used in this study, conditioned media from human fibrosarcoma cells (HT-1080; American Type Culture Collection, Rockville, Md) was used for a positive control. The cells were grown in an enriched media (10% fetal calf serum) to confluence and then were stimulated with phenyl methyl acetate (100 nmol/L) in minimal media. After 2 hours, the media were collected and concentrated. Protein concentrations were obtained using the method of Bradford, and the concentrated media were used as the positive control in both Western blotting and zymography.

Substrate zymography. Gelatin substrate zymograms were prepared by adding Type B bovine gelatin (Sigma, St. Louis, Mo) to a 10% sodium dodecyl sulfate-polyacrylamide gel at a final concentration of 1 mg/mL.[12] Twenty-microgram protein samples were electrophoretically separated using a minigel apparatus (BioRad, Hercules, Calif) under nonreducing conditions. Both Kaleidoscope and molecular weight size markers were used as standards (BioRad). The gels then were washed in 2.5% Triton X-100/50 mmol/L Tris solution twice for 20 minutes, followed by three 5-minute washes with a 50 mmol/L Tris/10 mmol/L CaCl/0.02% sodium azide, pH 7.8, solution. The gels then were incubated in the Tris-CaCl

buffer at 37°C for 16 hours, stained in Coomassie blue for 5 to 20 minutes, and destained with methanol. Zones of lysis were quantified with a scanning densitometer (Molecular Dynamics, Sunnyvale, Calif) and normalized to the HT-1080 digestion to compensate for gel-to-gel staining differences. Values are reported as the ratio between the actual value to the positive control (densitometric index [DI]). Kilodalton weight estimates were obtained using ImageFragment Software (Molecular Dynamics) and the molecular weight standards.

Western analysis. For Western immunoblotting, samples were prepared as above and electrophoretically separated onto standard 10% polyacrylamide Laemmli gels.[13] To correspond to the zymograms, all immunoblots were run under nondenaturing conditions. In addition, blots for MMP-2 and MMP-9 were separated under denaturing conditions as well. Monoclonal mouse anti-human antibodies to MMP-2, MMP-3, and MMP-9, tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), and α_2-macroglobulin were used as primary antibodies (Oncogene Science, Cambridge, Mass). Antibody binding was linear as confirmed in preliminary slot blot experiments using protein from both cell culture supernates and tissue specimens. Goat anti-mouse horseradish peroxidase-coupled antibodies were used as the secondary antibody (BioRad). Signal detection was performed using the Renaissance chemiluminescence kit (New England Nuclear, Boston, Mass) according to the manufacturer's instructions. Autoradiographs of the blots were obtained and subsequently analyzed using the densitometer as described above.

Immunohistochemistry. Immunohistochemistry was performed on 8-μm sections of vein as described previously.[14] Slides were initially washed in 0.1 mol/L PBS for 5 minutes followed by 2 3-minute washes in 0.05% borohydride in 0.1 mol/L PBS, finishing with a 5-minute wash in 0.3% Triton X-100 in 0.1 mmol/L PBS. Slides were again washed for 5 minutes in 0.3% Triton X-100 followed by a 2-hour incubation with the primary antibody in blocking solution (10% goat serum and 0.4 g of bovine serum albumin in 0.3% Triton X-100/PBS). Primary antibodies to MMP-2, MMP-9, and TIMP-1 were used. Slides were washed in 0.3% Triton X-100 and then incubated with indocarbocyanine-3-labeled goat anti-mouse IgG secondary antibody in blocking solution for 1 hour, followed by a series of washes: 5 minutes in 0.3% Triton X-100, twice for 2 minutes in 0.06% Triton X-100, and twice for 2 minutes in 0.1 mol/L PBS. Slides were then air dried and coverslip mounted with one drop of 50% glycerol. Specimens then were examined at 100× magnification using a fluorescent lens mounted on a Zeiss microscope. For quantitative analysis, images from the center of the vein wall were obtained in four different quadrants and were transferred to computer using a video camera where the fluorescence was quantified using MetaMorph software (Universal Imaging Co, West Chester, Penn). In addition, full wall images including lumens were obtained of specimens exposed to anti-MMP-9 antibodies (qualitative analysis). The 8-μm sections were also stained with both hematoxylin and eosin and trichrome stains.

Statistical analysis. All numeric data are reported as mean ± the standard error of the mean. Student's *t* test was used to compare densitometric indexes between patients with and without varicose veins, and the Fisher's exact test statistic was used to compare categorical data.

RESULTS

No significant differences in either age or gender distribution between patients with and without varicosities was identified.

Zymograms of vein protein homogenates demonstrated four discernible levels of proteolytic digestion. There was no difference in proteolytic activity between the samples from patients with and without varices at the three most prominent bands between 66 and 96 kDa. There was a significant decrease in proteolytic activity in veins from patients with varices at 59 kDa, although the least amount of proteolytic activity was noted at this level. The DI at this level for veins from patients without varicose changes was 1.97 ± 0.10 compared with 2.19 ± 0.03 in samples from patients with varices ($P < .05$).

Western immunoblots showed a significant decrease in MMP-2 immunoreactive protein content in veins from patients with varices, with a DI of 0.5 ± 0.1 versus 1.5 ± 0.3 in veins from patients without varices ($P < .05$; Fig 1). Conversely, immunoreactive TIMP-1 was significantly increased in veins from patients with varices (DI, 0.8 ± 0.1) compared with samples from patients without varices (DI, 0.2 ± 0.05, $P < .05$; Fig 2). There was no significant difference in immunoreactive protein between the two groups for MMP-3, MMP-9, α_2-macroglobulin, and TIMP-2.

Trichrome staining identified an increase in collagen deposition coupled with smooth muscle disorganization in sections from patients with distal varicose veins (Fig 3A, 3B). Qualitative analysis of immunohistochemistry preparations showed metal-

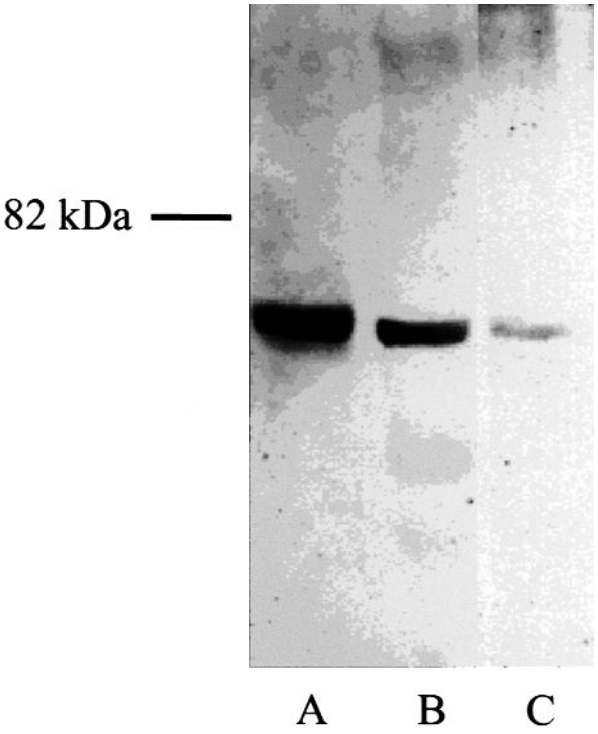

Fig 1. Representative immunoblot using primary antibody to MMP-2. *Lane A*, 20 μg of conditioned media from HT-1080 cells (positive control). *Lane B*, 20 μg of protein homogenate from patient without varices. *Lane C*, 20 μg of protein homogenate from patient with varicosities. Signal noted at approximately 66 kDa is consistent with MMP-2 and was decreased in the samples from patients with varicose disease.

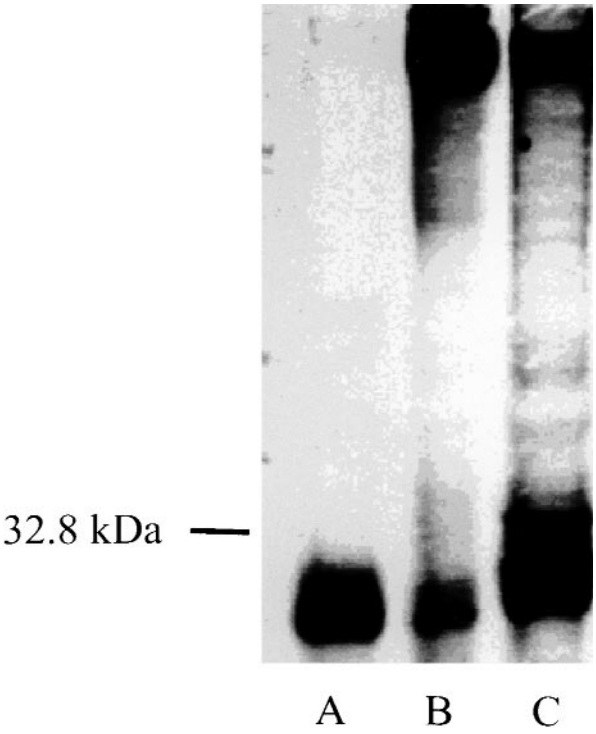

Fig 2. Representative immunoblot using primary antibody to TIMP-1. *Lane A*, 20 μg of conditioned media from HT-1080 cells (positive control). *Lane B,* 20 μg of protein homogenate from patient without varices. *Lane C,* 20 μg of protein homogenate from patient with varicosities. Signal noted at approximately 29 kDa is consistent with TIMP-1 and was increased in samples from patients with varicose disease.

loproteinase localization to the outer media and adventitia (Fig 3C, 3D). Quantitative analysis, however, did not reveal significant differences in immunohistochemical staining for MMP-2, MMP-9, and TIMP-1 between the specimens from patients with and without varices.

DISCUSSION

We demonstrated decreased immunoreactive MMP-2 protein and increased immunoreactive TIMP-1 protein in normal-appearing segments of the greater saphenous vein from patients with varicosities in the leg. Major differences in proteolytic activity were not detected between our study groups; however, a decrease in gelatinolytic activity was noted in the veins from patients with varices at 59 kDa, where the least amount of proteolytic activity was detected. Immunohistochemistry localized the enzymes of the metalloproteinase family to the adventitia, whereas the media was relatively devoid of these enzymes.

Our findings support recent suggestions that biochemical alterations of the vein wall accompany the development of varicosities. Shireman et al[10] reported an increase in urokinase-type plasminogen activator in varicose greater saphenous veins compared with normal veins and also noted an increase in tissue plasminogen activator at the level of the groin compared with the ankle. Because these activators of the plasmin fibrinolytic cascade may in turn activate latent MMPs, one might expect an increase in proteolytic activity at the saphenofemoral junction of patients with varicose veins.

Other circumstantial evidence support a proteolytic etiology of varicose veins. One report has shown a decrease in elastin and collagen content in varicose veins[15]; histologic evaluation has shown an increase in the deposition of degraded collagen, with disruption of the muscular bundles[6,7]; and ultrastructural examination has revealed fragmented collagen fibrils within medial smooth muscle cells,

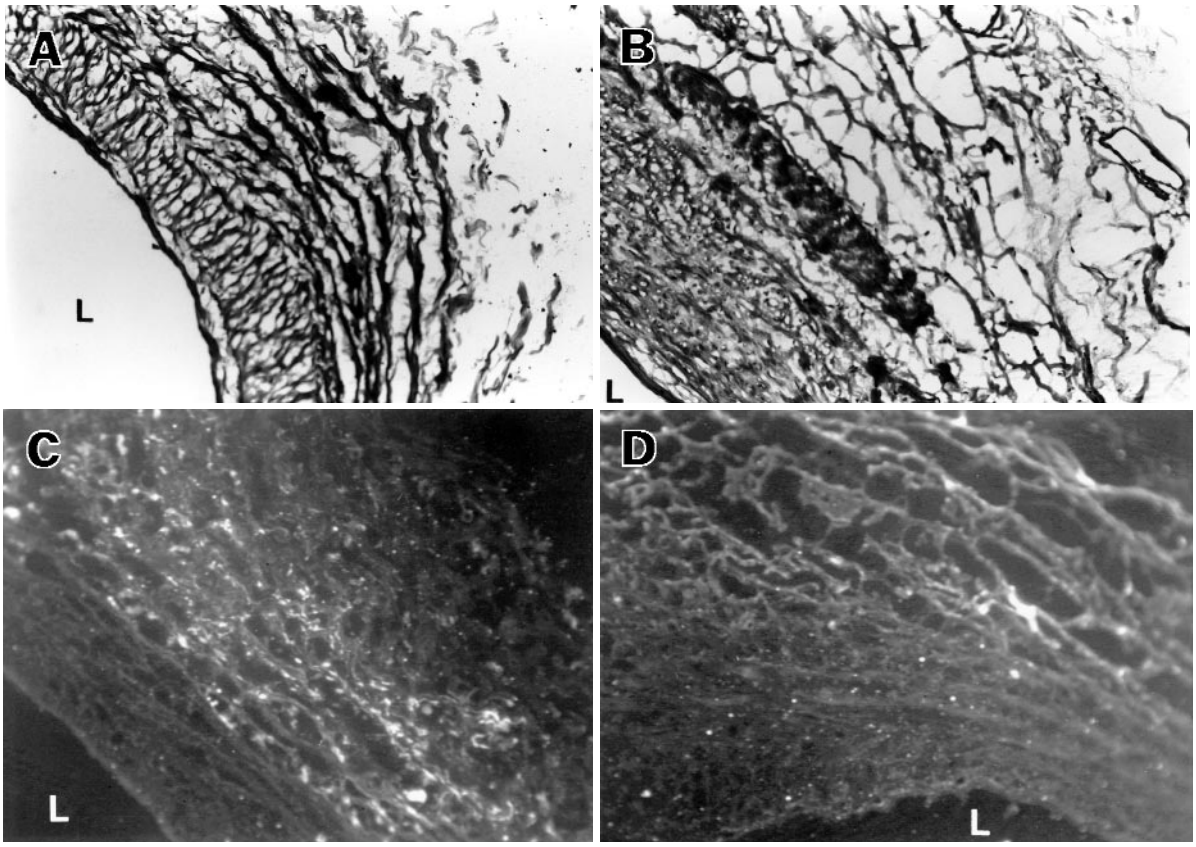

Fig 3. Photomicrographs and immunohistochemistry (magnification, 100×) of vein sections. *L*, Region of the lumen. **A**, Trichrome-stained section of saphenous vein near the groin from patient without varices. The media is composed entirely of well-organized smooth muscle cells. **B**, Trichrome-stained section of saphenous vein near the groin from patient with varices located lower in the leg. The media is infiltrated with collagen, and the smooth muscle architecture is disrupted. **C**, Immunohistochemical stain using monoclonal antibody for MMP-9 in section from patient without varices. Positive staining is noted only in adventitial and perivascular region. A paucity of staining is noted in the media. **D**, Immunohistochemical stain using monoclonal antibody for MMP-9 in section from patient with varices. A similar but less intense pattern of positive staining is noted compared with **c** despite infiltration of the media with collagen. Similar patterns of staining were noted with MMP-2 and TIMP-1 antibodies (data not shown).

implying that these cells are active in the reorganization of structural proteins in the vein wall.[4] These findings correlate with the morphometric data of Bocking and Roach,[9] who noted an increased distensibility of saphenous veins from patients with varicosities, and Clarke et al,[8] who found a significant reduction in vein wall elasticity in patients at high risk for the development of varicosities before the development of valvular incompetence. Lowell et al[16] noted a decrease in contractile capability of saphenous veins from patients with varicosities distally. Increased proteolytic activity of the vein wall could account for many of these findings.

However, we and others have been unable to demonstrate an increase in proteolytic activity within the walls of varicose veins. We did note a small but significant decrease in proteolytic activity in veins from patients with varices at 59 kDa, which may be due to activity of latent MMP-1 as noted by other investigators.[17] There was no significant difference in the majority of proteolytic activity that occurred at 66 and 84 kDa (corresponding to MMP-2 and MMP-9, respectively) on our zymograms. This agrees with the findings of Gandhi et al,[5] who were unable to detect a difference in proteolysis either by substrate zymography or radiolabeled substrate assay. Moreover, we detected a decrease in MMP-2 and an increase in TIMP-1 immunoreactive protein in specimens from

patients with varicosities; a finding that implies a predisposition toward decreased proteolysis in veins predisposed to varices compared with normal veins. A deficiency in proteolytic activity would also be consistent with the biochemical, histological, and ultrastructural changes noted above. Collagen deposition in the walls of varicose veins may result from the inability of the vein wall to completely degrade this extracellular matrix protein, predisposing the vein, leading to the morphometric and vasomotor changes noted.

Immunohistochemical analysis of our specimens localized the enzymes of the metalloproteinase family to the adventitia. This is consistent with the findings of Hach et al,[18] who noted a localization of fibrinolytic activity to the adventitia of normal and varicose veins, and with those of Newman et al,[19] who noted adventitial metalloproteinase expression in aortic aneurysms. In addition, Yamada et al[20] demonstrated clusters of mast cells in the adventitia, which may be a source for MMP protein.[19,20] However, George et al[21] did note the presence of MMP mRNA and protein in endothelial as well as adventitial microvascular endothelial cells. Although we used more specific antibodies in our study, we may have been unable to detect endothelial staining because of preparation artifact. In any event, both our study and that of George et al[21] confirm the paucity of MMP enzymes in the medial layers of normal and varicose veins.

Our study compared vein specimens of normal caliber obtained from patients with arterial insufficiency in the leg with those obtained from patients with distal varicose veins. The differences we observed between the two groups confirm the presence of metabolic alterations in the vein wall and suggest that these processes affect the entire vein. This study, however, has several limitations. Patients with chronic lower extremity ischemia may not represent the population at large with normal saphenous veins, and our findings may represent changes unique to this group of patients. This seems unlikely, however, given the excellent data available on the preoperative evaluation of the saphenous vein in patients with lower extremity ischemia. Veith et al[22] noted a subclinical presence of varices in only 5% of extremities studied with venography, and none of our patients were noted to have varices at operation.

We did not study actual varicosities because specimens from stab avulsion of varicose veins are usually damaged to the extent of compromising valid analyses. Rather, we examined saphenofemoral segments above the areas of varicose disease and have inferred that the alterations observed predispose to varicose degeneration. This inference is supported by the results of the study of Gandhi et al,[5] who noted similar connective tissue content and proteolytic activity in potential varicose veins (analogous to the specimens in our study) and in the varices themselves.

Zymography has inherent limitations insofar as activation of metalloproteinases by the sodium dodecyl sulfate present in the polyacrylamide gel causes falsely elevated estimates of proteolytic activity.[23] Thus, subtle differences in proteolytic activity may be undetectable and may explain our inability to demonstrate a decrease in proteolysis in veins from patients with varices despite an increase in immunoreactive MMP-2. Although the populations from which we obtained specimens were somewhat different, statistical analysis did not demonstrate a difference in age or gender distribution between our groups. Furthermore, both groups had a predominance of smokers, and the influence of tobacco use on our results cannot be determined.

We demonstrated alterations in metalloproteinase quantity and, to a lesser extent, activity in normal-appearing segments of saphenous vein from patients with varices. Decreased MMP-2 and increased TIMP-1 protein may establish an antiproteolytic milieu conducive to increased collagen deposition and eventual varicose degeneration. Further studies are needed to evaluate this process.

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